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No evidence of white adipocyte browning after endurance exercise training in obese men

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Running title: Exercise and adipocyte beiging in humans

Conflict of Interest Statement

The authors declare no competing financial interests in relation to the work described.

Abstract

Background/Objectives: The phenomenon of adipocyte 'beiging' involves the conversion of non-classic brown adipocytes to brown-like adipose tissue with thermogenic, fat-burning properties, and this phenomenon has been shown in rodents to slow the progression of obesity-associated metabolic diseases. Rodent studies consistently report adipocyte beiging after endurance exercise training, indicating that increased thermogenic capacity in these adipocytes may underpin the improved health benefits of exercise training. The aim of this study was to determine whether prolonged endurance exercise training induces beige adipogenesis in subcutaneous adipose tissues of obese men.

Subjects/Methods: Molecular markers of beiging were examined in adipocytes obtained from abdominal (AbSC) and gluteofemoral (GF) subcutaneous adipose tissues before and after six weeks of endurance exercise training in obese men (n=6, 37.3 ± 2.3 years, 30.1 ± 2.3 kg/m^2).

Results: The mRNAs encoding the brown or beige adipocyte-selective proteins were very lowly expressed in AbSC and GF adipose tissues and exercise training did not alter the mRNA expression of *UCP1, CD137, CITED, TBX1, LHX8* and *TCF21*. Using immunohistochemistry, neither multilocular adipocytes, nor UCP1 or CD137-positive adipocytes were detected in any sample. MicroRNAs known to regulate brown and/or beige adipose development were highly expressed in white adipocytes but endurance exercise training did not impact their expression.

Conclusions: The present study reaffirms emerging data in humans demonstrating no evidence of white adipose tissue beiging in response to exercise training, and supports a growing body of work demonstrating divergence of brown/beige adipose location, molecular characterisation and physiological function between rodents and humans.

Introduction

White adipose tissue (WAT) plays a critical role in regulating whole-body energy homeostasis by storing fatty acids during times of energy surplus, releasing fatty acids during increased metabolic demand, and by secreting adipokines to regulate an array of physiological functions [1-3]. Brown adipose tissue is located primarily in the supraclavicular and paraspinal regions of humans and influences energy homeostasis by regulating thermogenesis and increasing energy expenditure. Brown adipose tissue thermogenic activity can protect against obesity and insulin resistance in rodents [4], and in humans its function is inversely associated with body mass [5-7] and insulin sensitivity. This has stimulated interest in understanding how it develops in order to capitalize on its thermogenic potential for obesity-related therapies [8].

A third type of adipocyte, called 'beige' [9] or 'brite' [10] adipocytes, are interspersed within white adipose tissue in mice [11] and humans [9], and upon thermogenic stimulation are capable of developing morphological and functional resemblance to brown adipocytes, including thermogenic activity [12, 13]. Contemporary work in mice indicates that beige adipocytes exist in WAT depots and develop from unique precursor cells, and thus have a distinct developmental linage to white and classic brown adipocytes [14-16]. Adult humans are likely possess a range of classic brown through to beige adipocytes; serial tissue collection from superficial to deep cervical adipose tissue exhibit gene expression signatures representative of white, to beige, then to classic brown adipose, respectively. Importantly, the majority of the tissue considered brown adipose in humans, while heterogeneous, is likely beige [9, 16, 17], and precursors obtained from abdominal subcutaneous white adipose tissue can develop beige adipose tissue characteristics *in vitro* [18]. Thus, the adipocyte precursor population in white adipose tissue may give rise to beige adipocytes and/or factors in the adipose niche might drive inducible, functional beige adipogenesis.

Exercise training has beneficial effects on most systems of the body, improves metabolic functions and can contribute to weight loss. Recent rodent studies indicate that adipocyte beiging may be an adaptation to exercise training, which increases oxidative/thermogenic potential of white adipose tissue [19] and may thereby contribute to the anti-diabetic actions of exercise training. The secretion of muscle-derived proteins or metabolites has been proposed to induce beige adipogenesis, with potential exercise-responsive myokines including interleukin-6 [20], irisin [21], β-aminoisobutyric acid [22] and meteorin-like [23]. In addition, sympathetic nervous system activity to white adipose tissue and circulating catecholamines are increased during exercise [24], and chronic adrenergic stimulation can drive a 'browning program' in human adipose tissue, albeit under severe conditions such as patients with severe burns [25]. However, it remains to be established whether exercise training can induce beiging of white adipose tissue in humans, with most of the current literature reporting no effect of exercise training on the expression of genes that regulate thermogenic capacity [26-29]. Hence, the aim of this study was to determine whether prolonged endurance exercise training induces beige adipogenesis in subcutaneous adipose tissues of obese men, a population at risk of developing metabolic diseases.

Materials/Subjects and Methods

Subjects

The Monash University Human Ethics Committee approved all experimental protocols (CF12/3804, 2012001746). Six healthy, overweight males were recruited to participate in the study and were part of a larger cohort reported in a companion paper [30]. They provided written informed consent after a detailed explanation of the experimental procedures and associated risks. Participants were sedentary (<2 hours of purposeful exercise per week), were not taking any medications, were weight stable (for a specified duration), and reported no history of cardiovascular disease, impaired glucose metabolism, dyslipidemia or smoking. Their baseline characteristics were: age, 37.3 ± 2.3 years; body mass index,

30.1 ± 2.3 kg/m^2; waist circumference, 102 ± 4 cm; fasting blood glucose, 5.7 ± 0.2 mmol/L; fasting insulin, 60 ± 13 pmol/L. 'Brown-like' adipose tissue was obtained from the supraclavicular fossae within fat delineated by CT under direct PET-CT guidance as described previously [31], and paraffin-embedded sections provided for analysis. This individual did not participate in the exercise training program.

Experimental design

The experimental design has been described in detail previously [30]. Briefly, participants visited the laboratory on three occasions. On the first visit, their workload capacity was assessed by a graded cycling test to volitional exhaustion on a Lode cycling ergometer (Lode, Groningen, The Netherlands). Maximal heart rate (HR) was recorded, and maximal oxygen uptake (VO_2max) was estimated by using a validated equation on the basis of the maximum power output attained at volitional exhaustion. Participants with an estimated VO_2max > 45 ml/min/kg were excluded from the study. On the second visit, subjects returned for pre-training tissue sampling following an overnight fast. Participants voided, then lay supine for 15 minutes, after which a blood sample was obtained from an antecubital vein. Participants were then prepared for adipose tissue sampling. Xylocaine (2%, no epinephrine) was administered subcutaneously 3 cm from the umbilicus (abdominal subcutaneous, AbSC) and the upper portion of the buttocks (gluteofemoral, GF). A small incision was made at each site and adipose tissue was obtained as previously described [30]. Participants returned to the lab 36-48 h after their last exercise bout and tissue sampling was performed following the same procedures as described above.

Exercise training protocol

At least three days after adipose tissue excision, participants commenced six weeks of supervised aerobic exercise training, consisting of four sessions per week. Three sessions consisted of 30 minutes

of continuous exercise at an intensity of 75% HR max. Exercise duration was increased by five minutes every two weeks, such that participants were performing 40 minutes of exercise per session by the conclusion of the program. The fourth weekly session consisted of an interval session that was performed on a cycle ergometer, which consisted of three minute intervals at 85% HR max followed by three minutes at 65% HR max. This was repeated five times and progressed to seven intervals by the end of the program. A post-training volitional exhaustion test was performed on the last day of training.

Adipocyte Isolation and RNA extraction

Adipocytes were isolated in Krebs buffer (5 mM glucose, 5% bovine serum albumin, pH7.4) by collagenase digestion (1 mg/ml collagenase type 2; Sigma-Aldrich, St. Louis,MO, USA) for 30 min at 37°C in a rocking water bath [30]. RNA was extracted from isolated adipocytes using a mirVana miRNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA, USA). The RNA was reverse transcribed using a TaqMan® small RNA Assays kit (Life Technologies), and quantitative PCR (qPCR) was performed with commercially available TaqMan® probes for genes associated with general (*TBX1, LHX8*) and beige (*CITED, CD137, UCP1, PPARGC1AA*) adipogenesis and white adipocyte functions (CD36 and GPAT). 18s ribosomal RNA was used as an internal control. The qPCR was run on a BioRad T100™ Thermal cycler with the following parameters: 95^{o}C for 10 minutes followed by 40 cycles at 95^{o}C for 15 seconds and 60^{o}C for 60 seconds. The relative quantification was calculated using the ΔΔCt method.

Small RNA preparation and next generation sequencing

Small RNA libraries were generated using TruSeq Small RNA Sample Preparation Kit (Illumina, San Diego, CA, USA). Briefly, 3' and 5' RNA adapters were ligated to mature miRNAs. A reverse-transcription reaction was used to create single-stranded cDNA. The cDNA was then PCR amplified using a common primer and a primer that contained one of 48 index sequences, and then read on a

high-sensitivity DNA chip. The amplified cDNA construct was then gel purified in preparation for subsequent cluster generation.

miRNA analysis

Analysis of the next generation sequencing data has previously been described [30]. Briefly, next generation sequencing reads were quantified by using mirDeep 2 (Thermo Fisher Scientific) [32]. The read counts for each miRNA were then log-transformed and samples were visualized using MDS plots from the limma and gplots toolsets [33, 34]. Differential miRNA expression testing was performed using voom/limma [35] and multiple testing correction using Benjamini & Hochberg correction to control the False Discovery Rate.

Immunohistochemistry

Paraffin embedded sections of both AbSC and GFAT were stained with either CD137 (Abcam, ab203391), UCP1 (Abcam) (Abcam, ab10983) or haemotoxylin and eosin (Sigma; H&E). CD137 and UCP1 stained sections were counterstained with fluorescent mounting media containing DAPI (Vectashield). Serial sections from each treatment group were de-waxed and rehydrated using xylene and decreasing concentrations of ethanol. The sections were then washed in 0.1 M phosphate buffer (PB) and endogenous peroxidase activity was blocked using 1% hydrogen peroxide in 0.1 M PB for 15 minutes and subsequently washed again. The tissue was then immersed in a block solution containing 4% normal goat serum and 0.3 % Triton X-100 in 0.1 M PB for one hour. Following this step the tissue was incubated with the corresponding primary antibodies (1:1000 in block solution) for 24 h at 4^{o}C. Following primary antibody incubation, sections were washed and incubated with either goat anti-rabbit IgG (H+L) AlexaFluor 594 (1:400, Invitrogen) or goat anti-rabbit IgG (H+L) AlexaFluor 488 (1:400, Invitrogen) for fluorescent staining for 90 min at room temperature. Secondary antibody omission was used to determine specificity of the antibodies used. The CD137 and UCP1 sections were

thoroughly washed and coverslipped using antifade mounting media with DAPI (Vectashield). To visualize and image the sections a Zeiss microscope with a motorised stage and MicroFibre digital camera was used.

The haematoxylin and eosin staining was performed by the Monash University Histology Platform. Briefly, sections were de-waxed and rehydrated using xylene and absolute ethanol, washed with running tap water and immersed in Harris' haemotoxylin. Sections were washed before being submersed in acid alcohol, and washed sequentially with running tap water, Scott's tap water, and running tap water, after which the sections were immersed in Eosin (Sigma). Sections were then dehydrated using absolute alcohol and xylene. Sections were then coverslipped using antifade mounting media, then visualized and imaged as above.

Statistical analysis

All data are presented as mean ± SEM, unless otherwise specified. Statistical analysis was performed using paired Student's *t* tests or two-way ANOVA with Bonferroni correction for multiple comparisons where applicable. Statistical significance was set *a priori* at $P \leq 0.05$.

Results

Evidence of an exercise training effect

Six weeks of endurance exercise training had no effect on body mass (Pre: 94.2 ± 6.2 kg *vs.* Post: 92.3 ± 6.6 kg) or body mass index (Pre: 30.1 ± 1.6 kg/m^2 *vs.* Post: 29.5 ± 1.8 kg/m^2). Estimated VO_2 max (Pre: 37.7 ± 2.2 ml/kg/min *vs.* Post: 40.2 ± 3.5 ml/kg/min, $P<0.05$) and peak power output (Pre: 269 ± 18 W *vs.* Post: 280 ± 16 W, $P<0.05$) were increased following exercise training.

Brown and beige adipocyte-selective mRNAs are not, or are very lowly, expressed in subcutaneous adipose tissue

The mRNA encoding classic white adipose tissue proteins including *GPAT* and *FABP4* were abundantly expressed in both AbSC and GF adipose tissue before and after exercise training. The average threshold cycle (C_T) for the housekeeping gene *18S RNA* was 13.3 cycles whereas the mean C_T for *FABP4* and *GPAT* was 26.2 and 30.6, respectively (**Figure 1a**). Note that the relative expression of mRNA decreases as the C_T increases. The mRNA for *18S RNA, FABP4* and *GPAT* was detected in all samples except one (**Figure 1b**) and the sample with undetectable *FABP4* was excluded from further analysis. The mean C_T for the transcriptional coactivator *PPARGC1A* was 33.5 (**Figure 1a**). By contrast the mRNA encoding proteins associated with beiging such as *UCP1, CD137, TBX1 and LHX8* were either not detected, or were detected in very few samples (**Figure 1b**). Even in those mRNAs that were detected (*i.e. CITED*), the average C_T values were extremely high and frequently exceeded 35 cycles (**Figure 1a**), reaching 39.3 cycles in the case of *UCP1*. Of the mRNAs that were readily detected, only *PPARGC1A* expression was increased following endurance exercise training (**Figure 1c**; $P<0.05$).

Brown or beige adipocyte-selective miRNAs are not increased after exercise training

We next examined next-generation sequencing data of adipocyte samples to identify the presence of microRNAs (miRNAs) that are known to regulate brown or beige adipogenesis [36], and whether these miRNAs are regulated by exercise training. miRs-26a, -26b, -27b, -30c or -378a were detected in human white adipose tissue but expression levels were not altered by training (**Figure 2 a-e**).

No evidence of multilocular adipocytes or UCP1 or CD137 positive staining in white adipose tissue

Paraffin embedded AbSC and GF tissue sections were examined for general histology and stained with anti-UCP1 and anti-CD137 antibodies to identify the presence of brown or beige adipocytes. There was no evidence of multilocular adipocytes after H&E staining in any AbSC or GF adipose tissue section

(**Figure 3**). While the UCP1 antibody readily detected UCP1 in supraclavicular 'brown-like' adipose tissue **(Figure 4)**, UCP1 staining was not detected in any AbSC or GF adipose tissue sections (**Figure 3**). Similarly, CD137 was abundant in supraclavicular 'brown-like' adipose tissue **(Figure 4),** while there was no evidence of CD137 staining in AbSC or GF adipose tissue either before or after exercise training (**Figure 3**).

Discussion

Acute exercise and chronic endurance exercise training increase the expression of brown and beige adipocyte-specific genes, which induces a brown adipocyte-like phenotype in the white adipocytes of rodents [19, 37-39]. The major finding of the present study is that such molecular changes are not observed in human subcutaneous white adipocytes after six weeks of endurance exercise training in obese humans, suggesting that beiging of white adipose tissue in response to endurance exercise training may not be a phenomenon in this population.

While Norheim *et al* [27] previously demonstrated modest increases in UCP1 and TMEM26 mRNA expression following 12 weeks of endurance exercise training in men, it is more commonly reported that the mRNA expression of beige selective biomarkers are unchanged after endurance exercise training in humans [26, 28, 29, 40]. Consistent with this notion, we were unable to reliably detect the presence of several brown or beige adipocyte-selective genes including UCP1, CD137, TBX1, LHX8 and TCF21. Even when these genes were detectable, training did not alter their expression in either AbSC or GF subcutaneous adipocytes. Furthermore, we did not detect the presence of either multilocular adipocytes, nor UCP1 or CD137-positive adipocytes in white adipose tissue sections using immunohistochemical approaches that were concomitantly validated in human brown/beige adipose tissue.

MicroRNAs (miRNAs, miRs) are small non-coding RNAs that regulate gene expression networks [41] to modify biological functions including adipocyte differentiation [42, 43] and metabolism [42]. Several miRNAs have been shown to induce a brown adipocyte-like phenotype in white adipocytes [36]. Of the 12 miRNAs known to regulate brown and/or beige fat development, miRs-26a, -26b, -27b, -30c and -378 are highly expressed in human adipocytes [30], but their expression was not impacted by endurance exercise training. This new information provides another line of evidence indicating that endurance exercise training does not impact white adipocyte beiging in obese humans, which contrasts sharply with the murine literature demonstrating a pronounced beiging of white adipose tissue depots with exercise training (reviewed in [44, 45]). Another novel aspect of this study is that these key findings are relevant to anatomically distinct adipose tissue locations (*i.e.* subcutaneous abdominal and gluteofemoral) that are metabolically distinct [2] and may have divergent sympathetic innervation via the ganglionic neurons, which would be consistent with the traditional view of segmental sympathetic outflows to different sympathetically-innervated end-organs [46].

While the data presented herein provide evidence that exercise training does not induce adipocyte beiging, there are a number of potential limitations worth considering. Firstly, beiging in rodents occurs in discrete regions within specific adipose depots and while beige adipocyte-like features were not observed in any of the 24 independent adipose tissue samples, it is possible that our 'field of view' was too small and we did not sample from regions where beiging occurred. Notably, adipose tissue was sampled from two distinct anatomical locations in an attempt to circumvent this issue. Secondly, the post-training adipose tissue biopsy was collected 36-48 h after the last exercise session. While this time period was selected to detect stable changes in miRNA, mRNA and protein expression, it is possible that the molecular changes driving adipocyte beiging are transient and rapidly returned to pre-training levels. Nevertheless, we surmise that if beiging did occur then UCP1 protein expression and multilocular adipocytes should be detectable within this time frame. A third caveat to the interpretation

of the present data, and previous studies examining beiging / browning of human white adipose tissue, is that many of the beiging biomarkers were originally identified in murine studies [47], and thus may not be relevant to human biology. However, studies in isolated human adipocytes have demonstrated the presence and inducibility of the markers assessed herein [18]. Next, the exercise stimulus may have been insufficient to activate the transcription factors required for beige adipogenesis; however, the workload employed throughout the training program would almost certainly induce a marked increase in the putative drivers of adipocyte beiging including sympathetic nervous activity and myokine production (*e.g.* IL-6) [48, 49]. Reports from single-cell clonal analysis indicate that human preadipocytes expressing the cell surface marker CD29 produce adipocytes with high thermogenic potential [50]. These $CD29^+$ cells were isolated from subcutaneous and subplatysmal neck fat and although $CD29^+$ preadipocytes were previously isolated from abdominal, hip, and thigh regions of healthy humans [51], it is possible that this $CD29^+$ cell population is present in very low amounts in the adipose tissue depots assessed, which might explain the absence of beiging reported here. Finally, exercise-induced adipocyte beiging may be suppressed in obese individuals as previous studies have reported impaired beige adipogenesis in cultured adipocytes from overweight compared with lean humans [18] and reduced thermogenic potential in obesogenic mouse strains[13]. Arguing against this possibility, exercise training induced a robust increase in PGC1α mRNA in both AbSC and GF adipocytes and PGC1α is pivotal in the regulation of *UCP1* gene transcription.

The mechanisms explaining the differences in mice and humans in the beiging response to exercise remain unresolved. A number of factors lead to exercise-induced beiging in mice and include increased sympathetic nervous system activity; secretion of muscle-derived proteins such as irisin, myostatin, meteorin-like and brain-derived neurotrophic factor; and increased production and secretion of muscle-derived metabolites including lactate and β-aminoisobutyric acid (BAIBA) (reviewed in [45]). Notably, all of these exercise-induced changes occur in both species, making it unlikely that these can explain

the differences in the beiging response. Another possibility is that the beiging / browning pattern of adipocytes appears to be dependent on the adipose depot location, and this might differ between species. To this end, the expression of beige / brown adipocyte genes is unequivocally higher in subcutaneous compared to visceral adipose tissue in mice, which is the opposite pattern to humans [52]. Further studies are required to fully elucidate the molecular mechanisms underpinning the beiging of white adipocytes, which may then explain the species differences.

In conclusion, while beiging of subcutaneous adipose tissue occurs in response to exercise and other stimuli in rodents, the data provided in the present study demonstrates that no beiging occurs in two subcutaneous adipose tissue depots of obese men after prolonged endurance training. While these results do not preclude the findings that browning can occur in humans in response to other dietary and/or pharmacological treatments [8], we reaffirm emerging data in humans demonstrating no evidence of either supraclavicular BAT [53] or subcutaneous WAT beiging in adults in response to endurance exercise training.

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Figure legends

Figure 1: Expression of brown and beige genes in AbSC and GF adipocytes before and after 6 weeks of endurance exercise training. **A)** Average C_T values detected by qRT-PCR. 18s is the housekeeping gene (white fill), and is highly expressed. Highly expressed adipocyte genes (grey fill). Genes involved in browning and beiging (black fill) have high C_T values, translating to low expression. Dotted line denotes C_T=35. **B)** Positive identification of beige/brown and highly expressed white adipocyte-selective genes as determined by qRT-PCR. For panels A and B, n=24 consisting of n=6 participants, pre- and post-training for both AbSC and GF adipocytes. **C)** *PPARGC1A* mRNA content. # P = 0.04, training effect, pre- *vs.* post-training; n=6 per group. Data were assessed by 2-way repeated measures ANOVA. Data are presented as mean ± SEM for panels A and C.

Figure 2: Expression of adipocyte miRNAs involved in browning and beiging before and after 6 weeks of endurance exercise training. **A)** miR-26a, **B)** miR-26b, **C)** miR-27b, **D)** miR-30c and **E)** miR-378 before and after endurance exercise training in either AbSC or GF; n = 5. Data assessed by two-way repeated measures ANOVA with Bonferonni multiple comparisons tests. Data are presented as mean ± SEM.

Figure 3: Representative images of AbSC and GF adipose tissue after endurance exercise training. Adipose tissue sections stained with haemotoxylin & eosin (H&E) showed no evidence of multilocular adipocytes following exercise training and no evidence of UCP1 (red) or CD137 (green) positive staining. UCP1 and CD137 images were counterstained with DAPI to highlight the nuclei (blue). Scale bar represents 100 µm. CD137, tumor necrosis factor receptor family superfamily member 9; DAPI, 4',6-diamidino-2-phenylindole. UCP1, uncoupling protein 1.

Figure 4: Representative images of brown adipose tissue showing positive staining for UCP1 (red) or CD137 (green). Scale bar represents 100 µm.